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Increased soil nitrogen supply enhances root-derived available soil carbon leading to reduced potential nitrification activity

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ABSTRACT

Nitrogen (N) immobilisation by heterotrophic microorganisms is critical for reducing N losses from soils and ensuring a long-term supply of N to plants in grassland ecosystems. The supply of carbon (C) available to soil microbes may stimulate heterotrophic N immobilisation by reducing the availability of ammonium to autotrophic nitrifiers and, hence, for nitrification activity. The main source of available C to soils is rhizodeposition, but its effects on nitrification activity remain unclear as rhizodeposition differs between plant species and varying N availabilities. The aim of this work was to investigate the role of root-derived C on nitrification activity for five different grassland plant species. *Cichorium intybus* (chicory), *Lolium perenne* (perennial ryegrass), *Plantago lanceolata* (ribwort plantain), *Raphanus raphanistrum* and *R. sativus* (wild and cultivated radish), and an unplanted control were grown for nine weeks under controlled environmental conditions and treated either with a low (no urea-N) or a high rate of additional N (550 kg urea-N ha^{-1}). Plant biomass, water-extractable C concentration and ammonia-oxidising bacteria (AOB) abundance increased in the planted high N treatments. The high N addition to planted soils resulted in increased C available for microbial activity and led to decreased potential nitrification activity compared to those for the low N treatments. An increase in water-extractable C concentration was associated with a decrease in potential nitrification activity, suggesting that the increase in available C for microbial activity may have stimulated heterotrophic NH_4^+ uptake and thus N immobilisation.

This study highlights that N addition can be used to manipulate root-derived available C and, with the tight coupling of soil C and N cycling processes, can be used to identify management practices that will promote N retention and reduce losses from grassland soils.

1. Introduction

Many grassland ecosystems are limited by nitrogen (N) supply (LeBauer and Treseder, 2008), but management practices are needed to avoid excessive supply from fertiliser additions and livestock urine and dung that can lead to leaching losses and nitrous oxide emissions (Erisman, 2004; Galloway et al., 2008; Schlesinger, 2009). However, identifying effective management practices remains challenging because the mechanisms regulating N cycling and N retention capacities of grassland soils are poorly understood (de Vries and Bardgett, 2012, 2016; Weitzman and Kaye, 2016).

Nitrification of ammonium (NH_4^+) to nitrate (NO_3^-) is a critical step in the regulation of soil NO_3^- concentration, and thus the mobility and leaching susceptibility of N (Canfield et al., 2010). Managing conditions to limit nitrification is likely to result in reduced N leaching losses (Robertson and Vitousek, 2009). Nitrification typically increases when the available NH_4^+ supply exceeds plant N uptake (Robertson and Groffman, 2015), and this often occurs in intensively grazed grasslands with urine deposition, leading to high NO_3^- leaching losses (Crews and Peoples, 2005; Selbie et al., 2015).

A management option to balance N availability and plant demand is to increase immobilisation of excess soil N by soil heterotrophic microorganisms with the subsequent release of mineralised N when plant demand increases (Robertson and Vitousek, 2009). Heterotrophic microorganisms are more competitive for N than autotrophic nitrifying microorganisms (Booth et al., 2005; Dilly, 2005; Sayavedra-Soto and

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Arp, 2011), and the stoichiometric N demand of heterotrophs can be enhanced by increasing the supply of available carbon (C) substrates (Booth et al., 2005; Cleveland and Liptzin, 2007; Hart et al., 1994a; Sterner and Elser, 2002). Available C compounds that are microbially accessible can be derived readily from decomposed organic matter by microbial and enzymatic processes (Chen et al., 2014; Dungait et al., 2012; Marschner and Kalbitz, 2003). Increasing the available C supply (i.e., increasing the soil C:N ratio) has been shown to decrease autotrophic nitrification activity and reduce the risk of N leaching (Fisk et al., 2015).

Plantago lanceolata (ribwort plantain) and *Raphanus raphanistrum* (wild radish) have been identified for their potential to reduce soil nitrification activities (Massaccesi et al., 2015; O'Sullivan et al., 2017). However, it is unclear if this effect is attributable to the root release of allelochemicals, including biological nitrification inhibitors (BNI), that suppress nitrification activities by affecting nitrifying microbial or enzymatic processes, or to an increase in microbial N immobilisation resulting from increased C rhizodeposition (Carlton et al., 2019; Cong and Eriksen, 2018; Coskun et al., 2017a,b; Subbarao et al., 2015). Rhizodeposits can amount to 20 to 60% of net C assimilated by plants, equivalent to about 800 to 4500 kg C ha^{-1} annually (Neumann and Römheld, 2012), and the rate and composition of rhizodeposition varies between plant species and under different conditions, such as soil fertility (Badri and Vivanco, 2009; Bais et al., 2006; Jones et al., 2004; Nguyen, 2003). Previous studies have shown that plant N deficiency can reduce the exudation of amino acids, while N addition leads to an increase in rhizodeposition, including exudation of highly available C compounds, such as carboxylates and sugars (Bowsher et al., 2018; Carvalhais et al., 2011; Haase et al., 2007). Although it has been shown that soil heterotrophic microorganisms can rapidly assimilate rhizodeposited C (Bahn et al., 2013), the effects of C rhizodeposition on soil nitrification remain unknown, due to the variability in rhizodeposition between species, priming effects, and limited observations of the effects of N availability on rhizodeposits (Bowsher et al., 2018; Gärdenäs et al., 2011).

The aim of this study was to investigate the effects of increased available C supply from plant roots on the regulation of soil nitrification activities for different plant species. Our first hypothesis was that soil C availability would differ between plant species, and this would affect the abundance of ammonia-oxidising microorganisms and nitrification activities. The second hypothesis was that increased soil N supply would increase C availability, with subsequent decreases in nitrification activity. To test these hypotheses, we grew five grassland species with different root characteristics in microcosms with no and high addition of urea-N. We investigated changes in shoot and root biomass, shoot N concentration and content, soil pH, concentrations of total soil organic C and N, available C and mineral N, abundance of ammonia-oxidising microorganisms, and net nitrification activities associated with plant species, C availability and N addition.

2. Materials and methods

2.1. Site description, soil sampling and experimental design

Topsoil was collected to a depth of 150 mm from an irrigated ryegrass (*Lolium perenne* L.)-white clover (*Trifolium repens* L.) grassland at the Lincoln University Research Dairy Farm (LURDF), Lincoln, New Zealand (latitude 43.640° S, longitude 172.463° E; 14 m above sea level). The soil was a Templeton silt loam (Typic Immature Pallic soil (New Zealand Soil Classification) (Hewitt, 2010); Udic Haplustept (USDA) (Soil Survey Staff, 2014)) with a pH ($CaCl_2$) of 4.90 and an organic matter concentration of 44 g kg^{-1}. Soil mineral N concentrations comprised 13 mg NH_4^+-N kg^{-1} and 9 mg NO_3^--N kg^{-1}.

After collection, the soil was sieved (≤4 mm), homogenised and 980 g ± 1 g was packed into PVC microcosms (65 mm diameter, 240 mm depth) at a bulk density of 1.0 Mg m^{-3}. In the centre of each microcosm, we planted one seed of *Cichorium intybus* L. cv. 'Choice' (chicory), *Lolium perenne* L. cv. 'Prospect' (perennial ryegrass), *Plantago lanceolata* L. cv. 'Tonic' (ribwort plantain), *Raphanus raphanistrum* L. (wild radish), or *Raphanus sativus* L. cv. 'Saxa 2' (cultivated radish). An unplanted soil treatment was included as a control.

The microcosms were arranged in a completely randomised design in a plant growth chamber (Fitotron HGC 1514, Weiss Gallenkamp, UK). The plants were grown under controlled conditions: 16 h photoperiod, air temperature 22 °C, photosynthetically active irradiance of 500 µmol m^{-2} s^{-1} at canopy level and 70% relative humidity. Daily watering, supplemented with a weekly adjustment of gravimetric soil moisture content to achieve 60 to 80% water holding capacity (WHC), ensured sufficient water supply for plant growth and avoided drainage. Three weeks after the seeds were sown, a top dressing of superphosphate at a rate of 30 kg P ha^{-1} and ammonium sulphate ($(NH_4)_2SO_4$) at a rate of 50 kg N ha^{-1} was applied to each microcosm.

Plants were grown initially for five weeks to allow enough time for the root system to develop. Then, two N treatments were applied to the microcosms. Half of the microcosms received urea ($CO(NH_2)_2$) at a rate of 229 mg N kg $soil^{-1}$, applied in solution to simulate a N loading rate similar to that of a urine patch from dairy cattle of 550 kg N ha^{-1} (Selbie et al., 2015), hereafter referred to as the 'high N treatment'. The other half of the microcosms received the same volume of water but without urea. This treatment is hereafter referred to as the 'low N treatment', as low amounts of N from the initial soil N content and the early $(NH_4)_2SO_4$ fertiliser application were expected to have remained in the soil. Mineralisation of urea to NH_4^+, which could have limited NH_4^+ availability for nitrification, was considered negligible because urea in soils is typically hydrolysed within a few days after surface application (Burton and Prosser, 2001; Cabrera et al., 1991; Sigurdarson et al., 2018). In total, there were 48 microcosms, comprised of four replicate microcosms for each plant species and N treatment.

After applying the urea-N treatment, the plants were grown for another four weeks to ensure that the ammonia-oxidising microbial community had enough time to establish (Di et al., 2009). Following a total incubation time of approximately nine weeks after the seeds were sown, the microcosms were sampled destructively. After removing the soil and plants, soil samples were collected from the rhizosphere (soil adjacent to the roots) using sterilised spatulas and stored at −80 °C for subsequent DNA extraction. The shoots, roots, and bulk soil were separated carefully, the soil was sieved (≤4 mm), homogenised and stored at 4 °C in dark conditions until further processing.

2.2. Plant analyses and measurements of soil chemical properties

Plant shoots and carefully washed roots were dried at 60 °C for 72 h and then weighed. After grinding, the dried shoots were analysed to determine the C and N concentrations by dry combustion on an elemental analyser (Elementar Vario-Max CN Elemental Analyser, Elementar GmbH, Hanau, Germany). Shoot N contents were calculated by multiplying shoot N concentration with shoot biomass. Root C and N concentrations were not measured.

Gravimetric soil water content was determined from the decrease in mass from fresh soil after drying at 105 °C for 24 h. Soil pH was measured in 0.01 M $CaCl_2$ (1:2.5 w:v). For soil mineral N concentration, ammonium (NH_4^+) and nitrate (NO_3^-) were extracted with 2 M KCl (1:10 w:v) from a fresh subsample (Rayment and Lyons, 2011), and concentrations measured using flow injection analysis (FOSS FIAstar 5000, Foss Tecator AB, Hoganas, Sweden). Total organic C (C_t) and total N (N_t) were analysed by dry combustion on an elemental analyser (Elementar Vario-Max CN Elemental Analyser, Elementar GmbH, Hanau, Germany). For determining water-extractable C concentrations (C_{we}), 3.0 ± 0.05 g dry soil equivalent was eluted with 30 mL deionised water (1:10 w:v), centrifuged (3000 ×*g* for 20 min), filtered (0.45 µm) (Ghani et al., 2003) and analysed for dissolved organic C (Shimadzu TOC Analyser model 5000A with ASI-5000A, Shimadzu Oceania Pty Ltd., Sydney, Australia).

2.3. Carbon availability index

Calculations of the carbon availability index (I_C), which can be used to indicate the proportion of available organic C for microbial use (Parkinson and Coleman, 1991), were determined from the ratio of measurements of soil basal respiration rate (R) and substrate induced respiration rate (R_{SI}) (Cheng et al., 1996; Gershenson et al., 2009; Gutiérrez-Girón et al., 2015), using a modified method from Anderson and Domsch (1978). Briefly, 4.0 ± 0.05 g fresh soil was incubated with 0.3 mL deionised water in sealed glass vials in dark conditions at 25 °C for 1 h. The increase in CO_2 concentrations in the headspace between the start and end of the incubation period were measured and used to calculate R. This was then repeated after adding glucose at 10 mg g^{-1} (oven-dry soil basis) to a replicate soil sample for determination of R_{SI}. Gas samples from the headspace were taken with a syringe and injected through a CO_2-free air stream into a calibrated infra-red gas analyser (Model LI-7000, LI-COR Inc., Lincoln, NE, USA).

2.4. Potential nitrification activity

Potential nitrification activity (N_p), which represents the potential enzyme activity for ammonium oxidation (Kandeler et al., 2011), was estimated after destructive sampling by chlorate inhibition (Belser and Mays, 1980) following a modified procedure of Hart et al. (1994b). In brief, 15 ± 0.5 g fresh soil was placed into an Erlenmeyer flask containing a 100 mL mixture of 1.5 mM NH_4^+ and 1 mM phosphate buffer with a pH adjusted to 7.2 ± 0.1 and added 1.1 g L^{-1} sodium chlorate ($NaClO_3$) as a selective inhibitor of nitrite (NO_2^-) oxidation to nitrate (NO_3^-) (Belser and Mays, 1980). The soil slurry was incubated on a horizontal shaker (115 rpm) at 20 °C for 24 h. At 2, 6, 20, and 24 h after the start of the incubation, 10 mL aliquots with a consistent soil:solution ratio were removed from the flask and centrifuged for 10 min. The supernatant was mixed with sulphanilamide and N-1(1-naphtyl)ethylenediamine dihydrochloride (NED) for colour development (Griess reaction) and the NO_2^- concentration analysed colorimetrically at 540 nm. The slope of the linear change in NO_2^--N concentration during the incubation time was used to calculate N_p.

2.5. DNA extraction and real-time qPCR

Total genomic DNA was extracted in triplicate from 0.25 g ± 0.001 g rhizosphere soil using a NucleoSpin® Soil Kit (Macherey-Nagel, Düren, Germany). After homogenising the sample mechanically and lysing the cells with a buffer solution, the membrane-bound DNA was washed and extracted, eluted in 100 µL of 5 mM Tris/HCl (pH 8.5) and diluted with filtered and UV-sterilised ultra-pure water (1:10 v/v) to minimise potential inhibition of polymerase chain reaction (PCR). All samples were stored at −20 °C prior to real-time quantitative polymerase chain reaction (qPCR) analysis.

The abundance of the *amoA* genes for ammonia oxidisers were estimated from the qPCR analysis following procedures described by Di et al. (2009) using the primer pairs amoA-1F (5′-GGGGHTTYTACTGGTGGT-3′) (Stephen et al., 1999) and amoA R-i (5′-CCCCTCNGNAAANCCTTCTTC-3′) (Hornek et al., 2006) for bacterial *amoA* genes and Arch-amoAF (5′-STAATGGTCTGGCTTAGACG-3′) and Arch-amoAR (5′-GCGGCCATCCATCTGTATGT-3′) (Francis et al., 2005) for archaeal *amoA* genes. A robotic liquid handling system (CAS-1200, Corbett Life Science, Mortlake, Australia) was used to prepare the qPCR setup automatically and all reactions were performed on a Rotor-Gene™ 6000 real-time rotary analyser (Corbett Life Science, Mortlake, Australia). The thermocycling conditions of the qPCR and the specific primer combinations are shown in Table A.1 (Supplementary data). The total volume of 16 µL qPCR reaction mixture comprised a dilution of 8 µL 2× SYBR® Premix Ex Taq™ (TaKaRa, Japan), 0.4 µL of each primer, and 1.5 µL of diluted DNA extract. After each assay, a melting curve analysis ranging from 72 to 99 °C ensured that the melting temperature cycles only caused amplification of the targeted genes.

For standard curve development, the PCR amplicons from extracted DNA samples were purified with a PCR clean-up kit (Axygen Scientific, USA) and the PCR product concentration determined with a Qubit™ fluorometer (Invitrogen, USA). Standard curves were generated based on quantified PCR product in a series of 1:10 dilutions after real-time qPCR with the same thermocycling conditions (Table A.1, Supplementary data). Amplification efficiencies of 100% were obtained for both AOA and AOB *amoA* ($R^2 \geq 0.995$). Copy numbers for the *amoA* genes per unit mass of dry soil were calculated to estimate the abundances of AOA and AOB.

2.6. Statistical analyses

Plant and soil properties (root biomass, shoot biomass, pH, N_p, I_C, AOA and AOB abundance, shoot N concentration, C_t, C_{we}, N_t, NH_4^+-N, and NO_3^--N) were analysed using two-way factorial analysis of variance (ANOVA) with N application rate (2 levels) and plant species (5 levels) as factors, and included possible interactions. Where there were significant differences ($P < 0.05$) between group means, Tukey HSD post-hoc tests were conducted for multiple comparisons of all plant species and N application effects. Multiple comparisons were carried out with the 'multcomp' package in R to correct for multiplicity (i.e. control type I error) while making many simultaneous inferences (Hothorn et al., 2008). These differences were reported using confidence intervals (95% CI). Results from the unplanted control soil were excluded from statistical analyses that compared plant species effects but included for comparisons between planted and unplanted soils.

Prior to using the ANOVA, assumptions of normality of the residuals and homoskedasticity were assessed by visual inspection of residual plots and plots of predicted vs. observed values. If the variance violated the assumption of homoskedasticity, linear models were refitted using the sandwich estimator of the covariance matrix (Zeileis, 2004), which is consistent for cases of heteroskedasticity. Since I_C is derived from a ratio, I_C was log-transformed to correct for skewness (Koricheva et al., 2013). Spearman's rank correlations were used to investigate associations between two variables.

Potential relationships between N_p and soil variables were investigated using linear regression, initially selecting between competing and sometimes co-linear predictor variables and then comparing models with likelihood ratio tests (if nested) and Akaike Information Criterion (AIC). Residual checks were used throughout. Values of the parameters from these models were reported with 95% confidence intervals (95% CI). All statistical analyses were undertaken using R version 3.6.0 (R Core Team, 2019).

3. Results

3.1. Plant properties

Shoot biomass for *L. perenne*, *P. lanceolata*, and *R. raphanistrum* increased in the high N treatment by 440, 217, and 232% compared to the low N treatment, respectively, while there were no significant differences in shoot biomass between N treatments for *C. intybus* and *R. sativus* (Table 1). Root biomass was similar between N treatments for all species except for *L. perenne*, where the increase in root biomass was 283% in the high N treatment. Although *L. perenne* responded to the high N treatment with the largest increase in biomass compared to that for the other species, the concentration of N in its shoot overall remained unchanged. In contrast, shoot N concentrations for *C. intybus*, *P. lanceolata*, and *R. sativus* in the high N treatment increased by 251, 205, and 186% relative to those for the low N treatment, respectively.

The shoot N content, an estimate of N uptake for shoot biomass, increased significantly for all species in the high N treatment relative to the low N treatment. *Lolium perenne* showed the highest N uptake, with 47.5% of added N in the high N treatment measured in the shoot. In

Table 1
Shoot and root biomass, shoot N concentration and content of plant species under low and high N treatments. Data are mean values ± standard error, n = 4. Different letters indicate significant differences among species and N treatments ($P < 0.05$). Values for shoot N content are expressed as mg N per total mass of shoot dry matter.

Species	N treatment	Shoot biomass	Root biomass	Shoot N concentration	Shoot N content
		g dry matter	g dry matter	mg N g dry matter^{-1}	mg N shoot^{-1}
Cichorium intybus	Low	3.92 ± 0.81^{bd}	7.50 ± 1.31^{bc}	5.73 ± 0.61^{e}	21.8 ± 3.25^{d}
	High	5.37 ± 0.64^{bc}	11.90 ± 1.21^{b}	14.40 ± 0.79^{b}	76.5 ± 6.81^{a}
Lolium perenne	Low	2.00 ± 0.47^{de}	8.30 ± 3.49^{bc}	9.95 ± 2.01^{be}	17.0 ± 1.68^{d}
	High	8.80 ± 0.83^{a}	23.50 ± 4.46^{a}	12.30 ± 1.07^{bc}	106.5 ± 7.66^{b}
Plantago lanceolata	Low	2.94 ± 0.21^{cde}	9.21 ± 2.88^{bc}	6.95 ± 0.83^{de}	20.5 ± 2.72^{d}
	High	6.37 ± 0.34^{ab}	9.96 ± 1.22^{bc}	14.28 ± 0.57^{b}	90.5 ± 4.25^{ab}
Raphanus raphanistrum	Low	3.99 ± 0.93^{bd}	1.09 ± 0.27^{c}	6.13 ± 0.49^{de}	23.3 ± 4.03^{d}
	High	9.24 ± 0.92^{a}	4.47 ± 0.64^{bc}	7.28 ± 0.78^{cde}	69.0 ± 13.39^{abc}
Raphanus sativus	Low	0.45 ± 0.06^{e}	5.43 ± 0.37^{bc}	10.95 ± 1.03^{bd}	4.8 ± 0.25^{e}
	High	2.22 ± 0.38^{de}	8.60 ± 0.37^{bc}	20.40 ± 1.56^{a}	44.0 ± 5.29^{c}

contrast, *R. sativus* showed the lowest N uptake, with 19.4% of the N added in the high N treatment measured in shoot biomass, while plant uptake for *C. intybus*, *P. lanceolata*, and *R. raphanistrum* was 34.1, 40.3, and 30.7% of added N in the high N treatment.

3.2. Soil chemical properties

Soil NH_4^+-N and NO_3^--N concentrations in planted soils were higher in the high N treatment compared with those in the low N treatment by 0.705 mg kg^{-1} (95% CI, 0.430 to 0.981; $P < 0.001$) and 2.10 mg kg^{-1} (95% CI, 0.41 to 3.78; $P = 0.010$), respectively (Table 2). Mean NO_3^--N concentrations in the planted soils were significantly lower than those in the unplanted control soils. The NO_3^--N concentrations in planted soils were higher in the high N treatment under *C. intybus*, *L. perenne*, and *P. lanceolata* but slightly lower under *R. raphanistrum* and *R. sativus*, when compared with the low N treatment. These differences were only significant for *L. perenne*. The NH_4^+-N concentrations were higher in the high N treatment for all species, but only significantly higher for *L. perenne*.

Overall, C_t and N_t in planted soils were lower in the high N treatment by 1.93 g kg^{-1} (95% CI, −2.57 to −1.29; $P < 0.001$) and 0.161 g kg^{-1} (95% CI, −0.222 to −0.101; $P < 0.001$) compared to values in the low N treatment, respectively (Table 2), equivalent to a decrease of 3.17–11.7% for C_t and 1.45–11.9% for N_t.

Soil pH in the planted soils was 0.262 units (95% CI, −0.305 to −0.219; $P < 0.001$) lower in the high N treatments than that in the low N treatments (Table 2). Further, soil pH under *L. perenne* was 0.157 units higher (95% CI, 0.089 to 0.225; $P < 0.001$) than the soil pH for the other species.

3.3. Available soil carbon concentrations

The mean values for C_{we} in the planted soils was 23.3 μg g^{-1} (95% CI, 17.2 to 29.4; $P < 0.001$) higher in the high N treatment than that in the low N treatment (Fig. 1). There were no significant differences in C_{we} between plant species, whereas C_{we} in the unplanted control was significantly lower than the value in the planted soils for the high N treatment.

Measurements of I_C were 0.703 units (95% CI, 0.477 to 0.929; $P = 0.0109$) higher in the high N treatment than those in the low N treatment (Fig. A.1, Supplementary data), and I_C was correlated with C_{we} ($r = 0.434$, $P = 0.0055$). There were significant differences in I_C between plant species, but measurements of I_C were compromised by varying soil water contents of the samples ($r = 0.480$, $P < 0.001$).

3.4. Ammonia-oxidising archaea and bacteria abundances

The abundance of AOA *amoA* gene copies exceeded those of AOB in all treatments. The AOA abundance ranged from 5.7×10^6 to 13.6×10^6 *amoA* gene copies g^{-1} (Fig. 2A), while the AOB abundance was between 1.6×10^6 and 9.1×10^6 *amoA* gene copies g^{-1} (Fig. 2B). There were no significant differences in AOA abundance related to plant species or N treatment. In contrast, the high N treatment was associated with an increase in AOB abundance by 4.8×10^6 *amoA* gene copies g^{-1} (3.5×10^6 to 6.1×10^6; $P < 0.001$) compared to the low N treatment. There was no significant plant species effect.

3.5. Potential nitrification activity

Potential nitrification activity (N_p) decreased by 1.36 ± 0.14 μg NO_2^--N g^{-1} day^{-1} (95% CI, −1.64 to −1.07; $P < 0.001$) in the high N treatment relative to that for the low N treatment. This decrease was greatest

Table 2
Soil pH, concentrations of total organic carbon (C_t), total nitrogen (N_t), ammonium-nitrogen (NH_4^+-N), and nitrate-nitrogen (NO_3^--N) in the soils under the different plant species and N treatments. Data are mean values ± standard error, n = 4. Different letters indicate significant differences among species and N treatments ($P < 0.05$). Values for C_t, N_t, NH_4^+-N and NO_3^--N concentrations are expressed as per unit dry mass of soil.

Species	N treatment	NH_4^+-N	NO_3^--N	C_t	N_t	pH
		mg kg^{-1}	mg kg^{-1}	g kg^{-1}	g kg^{-1}	
Cichorium intybus	Low	0.56 ± 0.07^{de}	0.85 ± 0.56^{cde}	23.3 ± 0.3^{ac}	2.12 ± 0.03^{ac}	4.57 ± 0.03^{bc}
	High	1.57 ± 0.49^{ace}	4.10 ± 3.04^{bcde}	22.6 ± 0.1^{bc}	2.09 ± 0.03^{ac}	4.37 ± 0.04^{de}
Lolium perenne	Low	0.71 ± 0.02^{cd}	0.40 ± 0.03^{d}	24.0 ± 1.0^{ac}	2.18 ± 0.05^{ac}	4.74 ± 0.05^{a}
	High	1.65 ± 0.17^{a}	5.20 ± 1.65^{c}	22.3 ± 0.1^{bc}	$2.04 \pm 0.05b^{c}$	4.51 ± 0.03^{cd}
Plantago lanceolata	Low	0.55 ± 0.02^{e}	0.26 ± 0.02^{e}	25.1 ± 0.5^{a}	2.25 ± 0.05^{ab}	4.56 ± 0.02^{bc}
	High	1.05 ± 0.22^{ace}	2.86 ± 2.00^{cde}	22.3 ± 0.2^{bc}	2.12 ± 0.7^{ac}	4.34 ± 0.02^{e}
Raphanus raphanistrum	Low	0.94 ± 0.11^{bc}	0.79 ± 0.22^{cde}	24.0 ± 0.6^{ac}	2.30 ± 0.03^{a}	4.60 ± 0.02^{ac}
	High	1.53 ± 0.28^{ab}	0.73 ± 0.23^{cde}	22.4 ± 0.6^{bc}	2.07 ± 0.05^{bc}	4.30 ± 0.03^{e}
Raphanus sativus	Low	0.75 ± 0.15^{bce}	0.58 ± 0.09^{cd}	24.4 ± 0.4^{ab}	2.26 ± 0.03^{ab}	4.61 ± 0.03^{ac}
	High	1.24 ± 0.22^{ac}	0.48 ± 0.09^{de}	21.5 ± 0.2^{c}	1.98 ± 0.04^{c}	4.25 ± 0.04^{e}
Unplanted control	Low	0.73 ± 0.07^{bce}	12.43 ± 1.45^{b}	24.5 ± 0.8^{ab}	2.22 ± 0.06^{ab}	4.66 ± 0.04^{ab}
	High	0.96 ± 0.10^{bc}	69.21 ± 8.84^{a}	22.6 ± 0.6^{ac}	2.23 ± 0.05^{ab}	4.26 ± 0.02^{e}

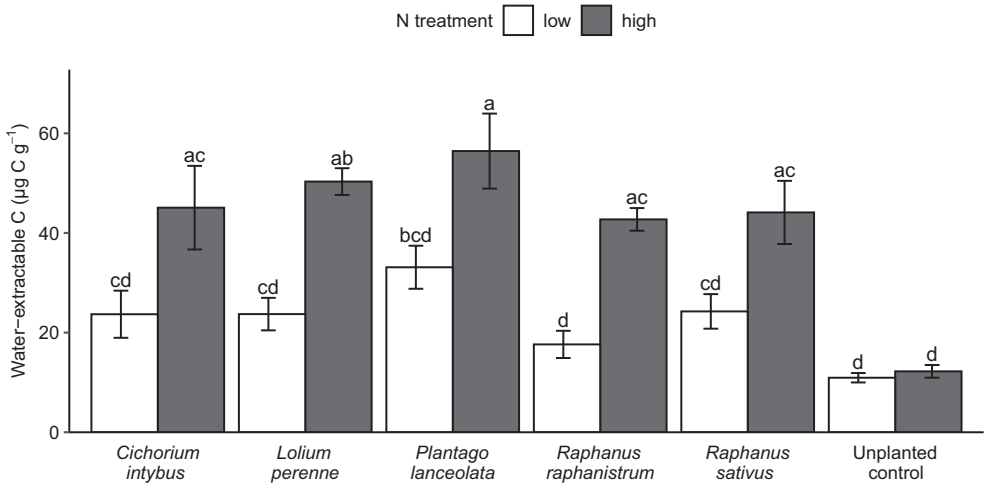

Fig. 1. Mean water-extractable C concentrations (C_{we}) in the soils for all plant species and controls with high and low N treatments. Error bars represent standard errors, $n = 4$. Different letters indicate significant differences among plant species or controls and N treatments ($P < 0.05$). Values are expressed as per unit dry mass of soil.

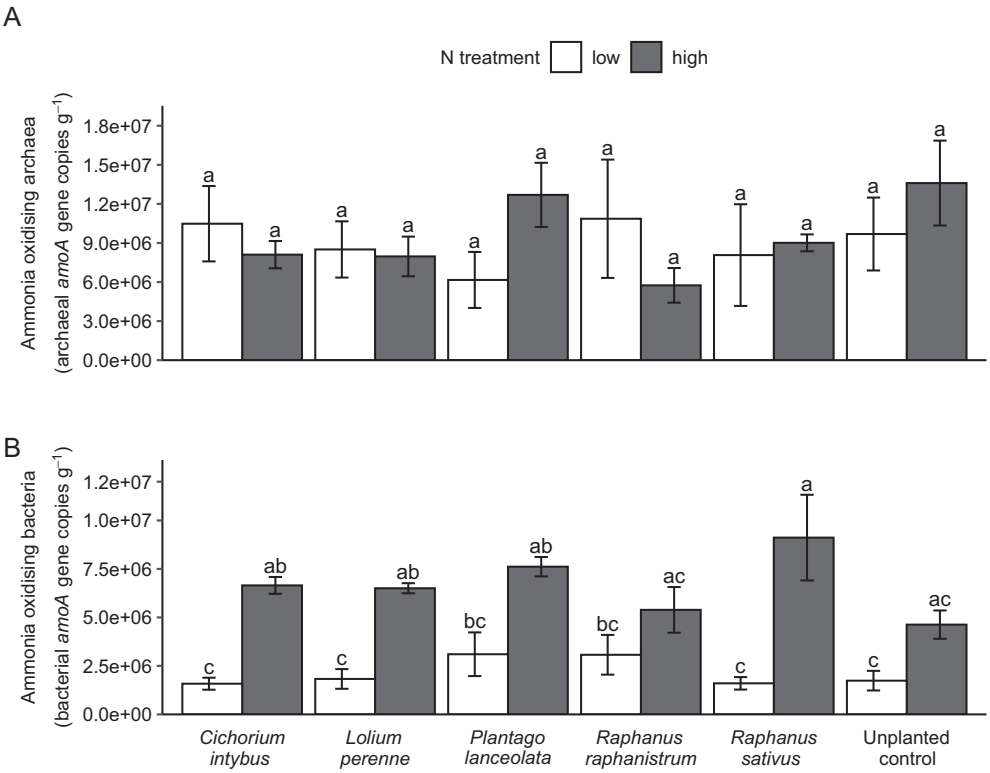

Fig. 2. Mean *amoA* gene copy abundances of ammonia oxidising archaea (AOA) (A) and bacteria (AOB) (B) in the soils under different plant species and N treatments. Error bars represent standard errors, n = 4. Different letters indicate significant differences among plant species and N treatments ($P < 0.05$). Values are expressed as per unit dry mass of soil.

for *P. lanceolata*, amounting to almost half the N_p (45.8%) in the high N treatment compared to that for the low N treatment (Fig. 3). For the other species, the decrease in N_p ranged from 26.8 to 33.0% in the high N treatments compared to that for the low N treatments.

There was a negative linear relationship between C_{we} and N_p (RMSE = 0.637 µg NO_2-N g^{-1} day^{-1}, $R^2 = 0.406$, $P < 0.001$), with a slope of −0.0343 µg NO_2^--N g^{-1} day^{-1} (95% CI: −0.048 to −0.021; $P < 0.001$) (Fig. 4). This negative relationship becomes evident when comparing the N_p:C_{we} ratio between the two N treatments, as C_{we} increased in the high N treatment (Fig. 5A) while N_p decreased (Fig. 5B) compared to values for the low N treatment. The N_p:C_{we} ratio was 66.7% lower in the high N treatment (0.064 µg NO_2^--N $µg^{-1}$C day^{-1}) than that in the low N treatment (0.19 µg NO_2^--N $µg^{-1}$C day^{-1}) (Fig. 5C).

4. Discussion

4.1. Soil nitrification potential and available carbon

The supply of available C has been shown to be an important driver for soil nitrification (Clarholm, 1985; Fisk et al., 2015; Knops et al.,

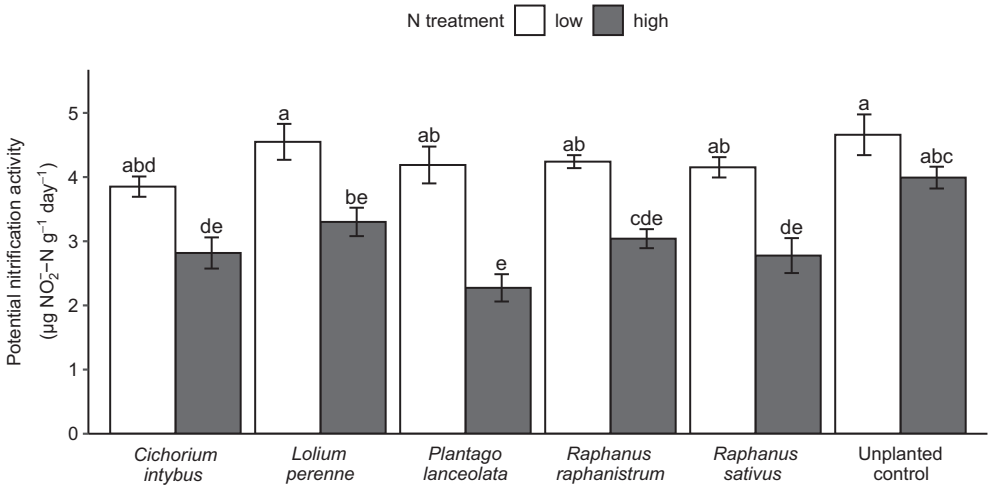

Fig. 3. Mean potential nitrification activity (N_p) in the soils under different plant species and N treatments. Error bars represent standard errors, n = 4. Different letters indicate significant differences among plant species and N treatments ($P < 0.05$). Values are expressed as per unit dry mass of soil.

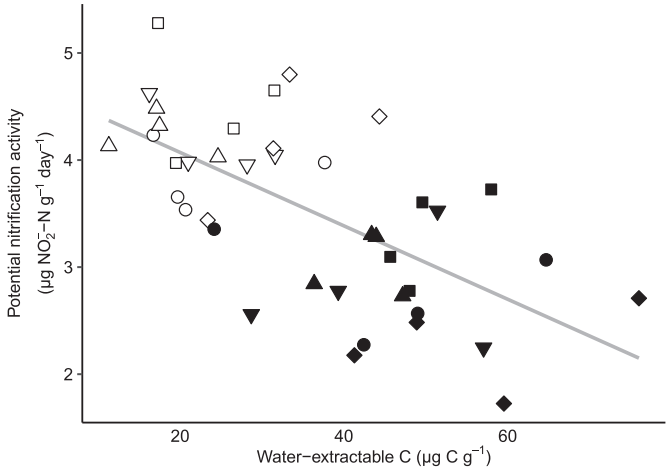

Fig. 4. Linear relationship between potential nitrification activity (N_p) and water-extractable C concentrations (C_{we}) for *Cichorium intybus* (○), *Lolium perenne* (□), *Plantago lanceolata* (◇), *Raphanus raphanistrum* (△), and *Raphanus sativus* (▽) with low N (white symbols) and high N (black symbols) treatments, $n = 40$. Values are expressed as per unit dry mass of soil. The linear regression is shown as a solid grey line (RMSE = 0.637 μg NO_2-N g^{-1} day^{-1}, $R^2 = 0.406$, $P < 0.001$).

2002; Paterson, 2003). Specifically, sufficient supply of microbially available C can stimulate growth of heterotrophic microbes, leading to increased immobilisation of NH_4^+ and its unavailability to autotrophic nitrifiers (Chen and Stark, 2000; Subbarao et al., 2006). Our findings support the assumption of a close coupling of root-derived available C and nitrification activities, showing a negative relationship between C_{we} and N_p across all plant species. However, we were not able to detect clear differences between plant species and so our findings do not support our first hypothesis. The overall higher C_{we} in planted than in unplanted soils implies that the C_{we} pool contains significant amounts of rhizodeposits. The correlation between C_{we} and I_C suggests that part of the C_{we} fraction was available for microbial use, supporting the assumption that soluble organic C can be used as an estimate for the available soil C fraction (Chantigny et al., 2014; Embacher et al., 2007; Marschner and Kalbitz, 2003; Pelz et al., 2005). We interpret the I_C data with caution as our measurements were confounded by differences in water contents of the samples (Parkinson and Coleman, 1991). Nevertheless, our results suggest that available C increased microbial C utilisation as indicated by I_C, which may have stimulated heterotrophic NH_4^+ immobilisation, leading to reduced nitrification activities. Even though soil NH_4^+-N concentrations were marginally higher in the high N than in the low N soils, this NH_4^+ may not be microbially available, since KCl-extractable NH_4^+-N was shown to be a poor indicator for microbially available N (Sawada et al., 2017; Scheu and Parkinson, 1995; Tiunov and Scheu, 1999; Vesterdal, 1998). Therefore, and because soil NH_4^+-N concentrations in all our treatments were low, it is possible that the soil microorganisms in our high N soils were limited by NH_4^+. This NH_4^+ limitation would increase for autotrophic nitrifiers if heterotrophic uptake stimulated by root-derived C further removed NH_4^+ by microbial N immobilisation. Since microbial N immobilisation was not measured in this study, we cannot with certainty attribute the observed decrease in N_p to an increase in heterotrophic N immobilisation induced by root-derived available C. However, our results support previous studies that have shown similar relationships between available C and N cycling (Bengtsson et al., 2003; Fisk et al., 2015; Gilliam et al., 2005; Szili-Kovács et al., 2007).

The increases in C_{we} in response to the treatments were likely attributable to higher rhizodeposition rates induced by N addition, because the increase was only apparent in the planted soils with the high N treatment and absent in the unplanted control soils (Henry et al., 2005; Nguyen, 2003; Warembourg and Estelrich, 2001). Rhizodeposits are the main source of available C to soils (Frank and Groffman, 2009; Pollierer et al., 2007; Sokol et al., 2019), and this was likely enhanced by the increase in shoot biomass resulting from N addition. Where the high N treatment led to an increase in shoot biomass, there was a significant increase in C_{we}, which suggests that increased leaf area, and possibly enhanced photosynthetic activity, led to higher rates of rhizodeposition of available C compounds (Dilkes et al., 2004; Högberg et al., 2001; Rogers and Humphries, 2000), supporting our second hypothesis. In addition to an overall increase in rhizodeposition, N addition can also contribute to changes in the composition of rhizodeposits (Bowsher et al., 2018). For example, high N availability can result in increasing exudation of amino acids (Carvalhais et al., 2011), which supply soil microorganisms with energetically and metabolically available C and N compounds (Drake et al., 2013).

In addition to rhizodeposition, N inputs may have led to increases in C_{we} by increasing the rate of soil organic matter decomposition, known as 'positive priming' (Conde et al., 2005; Hamer et al., 2009; Kuzyakov et al., 2000). Priming effects are not well understood (Blagodatskaya and Kuzyakov, 2008), with reports of negative or neutral effects with added N inputs to soil (Fornara et al., 2013; Kuzyakov et al., 2001; Ramirez et al., 2012). In our soil, the occurrence of positive priming is

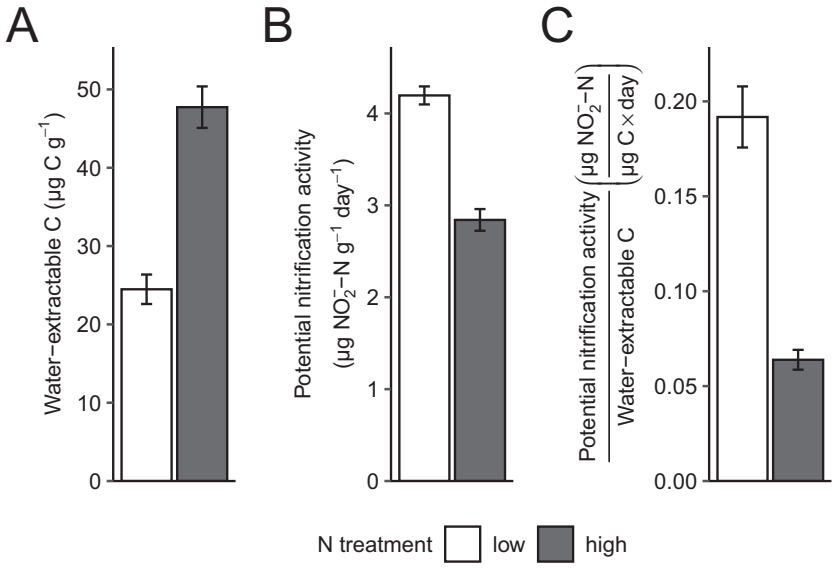

Fig. 5. Mean water-extractable C concentrations (C_{we}) (A), potential nitrification activity (N_p) (B) and ratio of potential nitrification activity over water-extractable C concentration (C) across all plant species grouped by N treatment. Error bars represent standard errors, $n = 20$. Values are expressed as per unit dry mass of soil.

more likely as C_t concentrations tended to decrease with N addition. Further, we measured lower C_t concentrations in the high N treatments for both the planted and unplanted soils, indicating that the effect cannot be attributed to the presence of roots alone. Similarly, Khalil et al. (2007) measured significant losses in C_t after adding N to unplanted soils. Soil organic matter decomposition in our soil was probably primed by stoichiometrically-regulated interactions between microbial decomposers (Chen et al., 2014; Guenet et al., 2010), specific root exudate compounds that chemically disrupt mineral-organic associations (Keiluweit et al., 2015), and a temporary pH increase following urea hydrolysis (Clough et al., 2010; Kelliher et al., 2005; Sherlock and Goh, 1984). However, the lack of a significant difference in both C_{we} and C_t between the high N and low N unplanted control soils suggests that priming effects had a minor influence on C_{we} compared to those from rhizodeposition. Nevertheless, the occurrence of a priming effect cannot be ruled out, and it is likely that soil organic matter decomposition contributed to the observed increase in C_{we}.

4.2. Soil nitrification potential and ammonia-oxidising microbial abundance

Nitrifying microbial communities in soils are typically sensitive to environmental changes, such as pH and N supply (Prosser and Nicol, 2012). In this study, the overall greater abundance of AOA relative to AOB in all treatments is probably related to the low pH of the soil, as acidic conditions typically favour AOA over AOB (Prosser and Nicol, 2012). This, and the differences in the response of the AOB and AOA populations to N application, support the current perception of niche differentiation between microbial populations in soils (Martens-Habbena et al., 2009; Prosser and Nicol, 2012). The lack of difference in the abundance of AOB and AOA communities between the planted and unplanted soils supports previous evidence that changes in the soil ammonia-oxidising microbial community are influenced dominantly by the available N supply, while the presence of plants and variation among plant species is less influential (Malchair et al., 2010a,b; Thion et al., 2016). Here, the N addition likely increased AOB biomass growth, as demonstrated previously (Di et al., 2009, 2010; Prosser and Nicol, 2012; Simonin et al., 2015), while an increase in the AOA community has been shown to be largely independent of soil N concentration, and can occur even with low N supply due to a high affinity for NH_3^+ (Levy-Booth et al., 2014; Martens-Habbena et al., 2009; Schleper and Nicol, 2010).

Ammonia-oxidising microbes are considered to drive soil nitrification (Prosser and Nicol, 2012), and a positive correlations between AOA or AOB abundance and soil NO_3^+-N concentrations or N_p has been shown (Di et al., 2009; Gubry-Rangin et al., 2010; He et al., 2007). Contrary to this, we showed a decrease in N_p in the soils with high N addition, together with a marginal increase in soil NH_4^+-N concentration, no difference in AOA abundance, an increase in AOB abundance and little evidence of a direct relationship between AOA or AOB abundance and N_p. As discussed in Section 4.1, we hypothesise that N_p was reduced by strong competition for NH_4^+ between heterotrophic microorganisms and autotrophic nitrifiers, influenced by root-derived available C. Unlike N_p, AOA and AOB gene copy abundance can reflect cumulative effects and antecedent conditions favouring increases in AOA and AOB communities may have occurred earlier and decreased subsequently until the time when destructive sampling took place. Yet, proportions of the AOA and AOB population may have persisted, including dead microbial cells, which would bias the measured microbial community (Carini et al., 2017; Dlott et al., 2015; Levy-Booth et al., 2014). In the context of this study, increased NH_4^+ availability and soil pH in the high N treatments would have likely declined throughout the weeks following the N application (Anderson et al., 2018; Clough et al., 2010; Kelliher et al., 2005) to the levels observed at the time of sampling, which could affect the AOA population and presumably decrease or even degrade the AOB population (Di et al., 2010; Frijlink et al., 1992; Lu and Jia, 2013). Although measurements made at the end of the experiment were not able to capture these dynamics, the measured AOA and AOB abundances most likely included dormant and dead DNA fragments, which would explain the lack of a relationship between AOA or AOB abundance and N_p in both this and previous studies (Hallin et al., 2009; Jordan et al., 2005; Rudisill et al., 2016; Wessén et al., 2010).

4.3. Soil nitrification potential and plant species effects

Overall, there was no significant plant species effect on N_p. However, the influence of C_{we} on N_p may have masked the potential effects of plant species. For example, previous studies that have shown a marginal plant species effect on N cycling suggest that other factors dominated N transformations, such as grazing regimes (Le Roux et al., 2003) and soil type (Groffman et al., 1996). Similar to our study, Stienstra et al. (1994) found no significant plant species effect on nitrification activities in a grassland system, but an overall reduction in nitrification when plants

were present, which they attributed to enhanced NH_4^+ immobilisation.

The shoot N contents indicated that the plant species were able to take up large amounts of N from the soil, so this may have limited N supply to soil microorganisms. Soil microorganisms are generally more competitive for N uptake than plant roots, but plants benefit from the fast turnover time of microbial biomass that supplies available N (Kuzyakov and Xu, 2013). In our study, soil NH_4^+-N concentrations were low in all treatments but slightly higher in the high N treatments compared to the low N treatments. Although this may suggest that plant roots were not more NH_4^+ limited in the high N soil than in the low N soil, it is possible that this NH_4^+ is bound to clay minerals and thus not easily available for plant and microbial uptake (St. Luce et al., 2011; Vesterdal, 1998). Taken together, it is unlikely that root uptake induced NH_4^+ limitation that led to reduced N_p in the high N treatment.

Another possible explanation for the decrease in N_p in the high N treatments is a potential increase in soil respiration in response to the high N addition (Barnard et al., 2004). Higher respiration would decrease available oxygen, which would limit nitrification (Grundmann et al., 1995). Although no respiration measurements were made, an increase in soil respiration following N addition is possible, as reported in other temperate grassland systems (Craine et al., 2001; Graham et al., 2014). This is supported by the increased root biomass in the high N treatments, because an increasing root N concentration can be related to enhanced root respiration rates (Bahn et al., 2006).

Even though we did not measure root N concentration, the increase in shoot N concentrations for all species in the high N treatments indicates that N was taken up by the plants and thus concentrations may have increased in all plant components. The likely increase in photosynthesis with increased shoot N content could have led to increases in root C concentrations, where the root C:N ratio would remain unchanged. In support of this, Cong and Eriksen (2018) reported that the root C:N ratio of *L. perenne* decreased after the addition of 250 kg N ha^{-1}, while that of *P. lanceolata* remained constant. They related the overall low root C:N ratio of *P. lanceolata* to increased labile C inputs into the soil, which supports our observations of enhanced C_{we} for *P. lanceolata*. However, the lack of significant differences in C_{we} between species within each N treatment does not indicate whether potential differences in root C:N ratios may have affected N_p.

Some studies have observed inhibitory effects on soil nitrification associated with specific plant species, among them *R. raphanistrum* (O'Sullivan et al., 2017) and *P. lanceolata* (Dietz et al., 2013; Luo et al., 2018; Massaccesi et al., 2015). In this study, we found no differences in N_p between any of the species tested. While attempts have been made to relate low nitrification activities to the root-release of biological nitrification inhibitors (BNI) that inhibit nitrification specifically (Carlton et al., 2019; Luo et al., 2018; O'Sullivan et al., 2017), the influence of root-derived available C on NH_4^+ immobilisation has often been overlooked. To our knowledge, no BNI compounds have yet been identified for any of the plant species used in this study. However, their possible presence and influence on N_p cannot be excluded. Future research investigating the mechanisms of nitrification inhibition by specific plant species is needed to determine the relative effects of both C and BNI compounds on nitrification.

5. Conclusions

We have provided evidence that the addition of N to grassland plant species increased soil C availability, which is likely attributable to enhanced rhizodeposition. The increased root-derived C was probably available for heterotrophic microbial growth and this may have reduced potential nitrification activity. The findings support growing evidence that the risk of N leaching from soils is greatest under conditions of low available C supply to soil from plants, for example during winter condition or following biomass harvest when photosynthetic activity is low. Maintaining continuous plant cover and active growth is important for increasing plant N uptake and rhizodeposition of available soil C that will lead to increased ecosystem N retention, and reduced N leaching and gaseous losses. Further studies in field conditions are needed to support the development of management practices to increase inputs of available C to soils and reduce N losses.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2020.103842.

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